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Intrafollicular transfer of fresh and vitrified immature bovine oocytes

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ABSTRACT

Embryo production by intrafollicular oocyte transfer (IFOT) represents an alternative for production of a large number of embryos without requiring any hormones and only basic laboratory handling. We aimed to (1) evaluate the efficiency of IFOT using immature oocytes (IFIOT) and (2) compare embryo development after IFIOT using fresh or vitrified immature oocytes. First, six IFIOTs were performed using immature oocytes obtained by ovum pickup. After insemination and uterine flush for embryo recovery, 21.3% of total transferred structures were recovered excluding the recipient's own oocyte or embryo, and of those, 26% (5.5% of transferred cumulus–oocyte complexes [COCs]) were morula or blastocyst. In the second study, we compared fresh and vitrified-warmed immature COCs. Four groups were used: (1) fresh immature COCs (Fresh-Vitro); (2) vitrified immature COCs (Vit-Vitro), with both groups 1 and 2 being matured, fertilized, and cultured *in vitro*; (3) fresh immature COCs submitted to IFIOT (Fresh-IFIOT); and (4) vitrified immature COCs submitted to IFIOT (Vit-IFIOT). Cumulus–oocyte complexes ($n = 25$) from Fresh-IFIOT or Vit-IFIOT groups were injected into dominant follicles (>10 mm) of synchronized heifers. After excluding one structure or blastocyst, the recovery rates per transferred oocyte were higher ($P < 0.05$) for Fresh-IFIOT (47.6%) than for Vit-IFIOT (12.0%). Blastocyst yield per initial oocyte was higher ($P < 0.05$) for Fresh-Vitro (42.1%) than for Fresh-IFIOT (12.9%). Vit-Vitro presented higher ($P < 0.05$) embryo development (6.3%), compared to Vit-IFIOT, which did not result in any extra embryo. Although IFOT did not improve developmental competence of vitrified oocytes, we achieved viable blastocysts and pregnancies produced after IFIOT of fresh bovine immature oocytes. Further work on this technique is warranted as an option both for research studies and for clinical bovine embryo production in the absence of laboratory facilities for IVF.

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1. Introduction

The ability to preserve female gametes is an integral part of assisted reproductive techniques as it has a great impact on animal conservation programs, animal breeding programs, and human fertility preservation. [1,2].

To date, the ability of cryopreserved oocytes to achieve later embryonic development is unsatisfactory in most domestic animals. Among the factors responsible for the severe damage caused during cryopreservation [3–10], the high cytoplasm lipid content and the membrane

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phospholipid composition [11] are often described as responsible for these injuries.

In the last decade, many efforts have been made to increase the vitrification efficiency for farm animal oocytes [1,8], including the use of stabilizing agents for the cytoskeleton [12], antioxidants [13], lipolytic agents [14,15] and plasma membrane modifiers [4,16]. However, no significant improvements have been obtained.

Although no difference in oocyte diameter is evidenced between cattle and humans [11], Cobo et al. [17] and Chang et al. [18] reported that human embryonic production from vitrified oocytes is similar to that obtained from fresh oocytes. These observations indicate that the cell size problems can be overcome and may not be the biggest problem to oocyte cryopreservation. Therefore, we hypothesized that the method of maturation could be an important factor in the poor survival of *in vitro*–matured bovine oocytes subjected to vitrification. In fact, it has been shown in cattle that IVM leads to different lipid and mitochondrial behavior patterns in matured oocytes which can, in turn, alter lipid metabolism affecting cryoresistance [19] However, we found no improvement after vitrification of bovine *in vivo*–matured oocytes (3.6% blastocysts after warming and IVF) in comparison with vitrification of *in vitro*–matured oocytes (2.8%–4.3%) [20].

The *in vitro* system is well known to induce a number of biochemical and morphological changes, such as higher lipid accumulation [21], changes in O_2 consumption [22], and changes in gene expression [23] In addition to the environmental conditions themselves, several specific molecules produced in the reproductive tract of the cow have been shown to be essential for the embryo. Therefore, the development of one entire *in vivo* system, which is the gold standard for embryo production, could be favorable for the vitrified oocytes, allowing them to develop at a higher rate than the *in vitro* environment.

One option to provide *in vivo* conditions for the vitrified oocytes would be to place then back into the female tract by intrafollicular oocyte transfer (IFOT), which was first proposed in baboons and cattle [24] and was then used successfully in mares [25]. This technique would allow the injection of immature oocytes to preovulatory follicles; thus, those oocytes could be matured, fertilized, and cultured in the female tract until the blastocyst stage, when embryos can be recovered by uterine flushing. Hinrichs and DiGiorgio [25] were the first to perform oocyte injection in mares; they injected the oocytes into the follicle with a needle that passed through a cannula placed on the abdominal wall, to puncture the follicle. Subsequently, a transvaginal ultrasound guide was used to localize and guide the needle to perform the injection in mares [26], cattle [27], and even in humans [28]. The first pregnancy and live healthy birth was only recently reported after injection of matured oocytes into the preovulatory follicles of recipients [29]. To the best of our knowledge, there has been no report of pregnancies after transfer of IFOT-derived embryos, when intrafollicular immature oocyte transfer (IFIOT) was used. Also, there is no report of using vitrified and warmed immature oocyte for IFIOT. Our hypothesis was that the entire *in vivo* system using IFIOT would positively affect the oocyte after vitrification and warming. First, a preexperiment was performed to evaluate the feasibility and quality of IFIOT-produced embryos. After this, we compared the developmental ability and embryo outcomes of fresh and vitrified/warmed oocytes submitted to *in vivo* (IFIOT) or *in vitro* system.

2. Materials and methods

Unless otherwise specified, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cryotop devices were acquired from Dibimed-Biomedical Supply (S.L. Valencia, Spain).

2.1. Ethics

All the experiments, including the preexperiment, were conducted according to Brazilian laws for animal ethics and health research and were approved by the Institutional Animal Care and Use Committee (Embrapa) protocol (process number 005/2015).

2.2. Experimental design

First, a preexperiment was performed to verify the viability of using immature oocytes for intrafollicular transfer. We performed ovum pickup once a week for 3 weeks on five Gir cows (*Bos taurus indicus*). The recovered cumulus–oocyte complexes (COCs) with intact cytoplasm and at least three cumulus cell layers were then used for IFIOT (n = 10–22 per transfer, total of six transfers), in which Nellore (*Bos taurus indicus*) heifers were used as COCs recipients. Note that only recipients that had one dominant follicle at the moment of injections were used. Frozen-thawed semen from a single Gir bull was used for artificial insemination after IFIOT. Subsequently, we recovered bovine morulae and blastocysts by uterine flushing 8 days after IFIOT, corresponding to the seventh day of embryo development (D7). Blastocysts graded as I or II according to International Embryo Technology Society (IETS) were immediately transferred to previously synchronized Nellore heifers, and pregnancy was assessed by ultrasound of the uterus 60 and 90 days after embryo transfer.

Second, COCs were recovered from slaughterhouse ovaries and were selected and distributed into four treatment groups: (1) fresh immature oocytes (Fresh-Vitro); (2) vitrified/warmed immature oocytes (Vit-Vitro), with both group 1 and 2 subjected to IVM, IVF, and IVC; (3) fresh immature oocytes submitted to IFIOT (Fresh-IFIOT); and (4) vitrified/warmed immature oocytes submitted to IFIOT (Vit-IFIOT). A total of 40 Nellore heifers (*Bos indicus*) that were approximately 40-month old and with similar body conditions were used as oocyte recipients. In each replicate, five heifers were previously synchronized and were artificially inseminated with frozen-thawed semen from the same Nellore bull used for the *in vitro* embryo production. At D7 of development (8 days after IFIOT), embryos produced following IFIOT were recovered by uterine flushing and were morphologically evaluated and compared with their *in vitro*–produced counterpart. Expanded blastocysts from all groups were then stained, for assessment of total

cell number and apoptotic cell number. To evaluate whether the follicular injection would affect the CL formed after ovulation, the CL was examined immediately after uterine flushing with the aid of a Color Doppler ultrasound for size and vascularization.

2.3. Ovum pickup

The ovum pickup was performed as described previously [20]. In summary, an ultrasound device (Aloka SSD 500, Japan) coupled to a micro convex sector transducer of 7.5 MHz (Aloka, UST 9125, Japan) was used. All the follicles between 3 and 8 mm were punctured. The recovered COCs were selected under a stereomicroscope (Zeiss–temi SV6, Germany) and were immediately subjected to IFIOT.

2.4. Oocyte recovery from slaughterhouse ovaries

Ovaries (*Bos indicus*) were collected immediately after slaughter (Qualimaxima, Brasilia-DF, Brazil). The time for ovaries transportation from the slaughterhouse to the laboratory was around 1 hour. They were transported in saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (100 g/mL) at 35 °C. Cumulus–oocyte complexes were aspirated from 3- to 8-mm-diameter follicles with an 18-ga needle and pooled in a 15-mL conical tube. Cumulus–oocyte complexes were recovered and selected in holding medium (HM) consisting of HEPES-buffered TCM-199 (Gibco BRL, Burlington, ON, Canada) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA). Only COCs with homogenous cytoplasm and at least three layers of cumulus cells were used for the experiments.

2.5. In vitro maturation

Selected COCs were washed and transferred in batches of 25 to 30 complexes to a 200-μL drop of maturation medium under silicone oil and were incubated for 22 hours at 39 °C with 5% CO_2 in air. The maturation medium consisted of TCM–199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 0.01 IU/mL of FSH, 0.1 mg/mL of L-glutamine and antibiotics (amikacin, 0.075 mg/mL).

2.6. IVF and IVC

After maturation, COCs (in groups of 25–30) were transferred to a 200-μL drop of fertilization medium. Frozen-thawed semen from a single Nellore bull previously tested in our laboratory for IVF was used for fertilization; the same bull was also used for artificial insemination. Motile spermatozoa were obtained by the Percoll method [30] and were added to the droplets containing COCs at a final concentration of 1×10^6 spermatozoa/mL. The fertilization medium was Tyrode's albumin lactate pyruvate [31] supplemented with penicillamine (2 mM), hypotaurine (1 mM), epinephrine (250 mM), and heparin (10 μg/mL). Spermatozoa and oocytes were coincubated for 18 hours at 39 °C with 5% CO_2 in air. The day of *in vitro* insemination was considered to be Day 0. After coincubation, the presumptive zygotes ($n = 25–30$) were washed, transferred to 200-μL drops of SOFaaci medium [32] supplemented with 2.77 mM of myoinositol and 5% fetal bovine serum, and were cultured at 39 °C and 5% CO_2 in the air for 8 days. Embryo development was evaluated on Day 2 after insemination for cleavage and on Day 7 for the blastocyst rate.

2.7. Estrus synchronization and IFIOT

To increase the chance of a preovulatory follicle at the day of IFIOT, heifers were submitted to an estrus synchronization protocol by receiving an intravaginal progesterone implant (Primer Tecnopec, São Paulo, Brazil) associated with the application of 2 mg of estradiol benzoate (intramuscular [im], RIC-BE, Brazil). Eight days later, animals received an injection of 2 mL (im) of prostaglandin F2α (0.150 mg d-cloprostenol [RARS Prolise SRL, Argentina]), together with the removal of the progesterone implant; 1 mg of estradiol benzoate (im) was administered on the following day. The injection of oocytes was performed 52 to 58 hours after progesterone removal, which occurred approximately 4 to 6 hours after the estrus onset. On the day of the IFIOT, the recipients were examined, and only those presenting a single preovulatory follicle $\geq$10 mm in diameter and with initial vascularization (as defined in the following) were used.

For the intrafollicular injection, we use a transvaginal guide (WTA, Brazil), containing a convex ultrasound probe of 7.5 MHz (Aloka, Japan). The transvaginal guide contained a mandrill mounted in a closed system, having at one end an insulin syringe and a 27-ga needle at the other end. First, the entire system was filled with warmed PBS, and at the end, an air bubble was pulled into the system. Then, all the 25 COCs were loaded into the needle with 60 μL of frozen-thawed follicular fluid by negative pressure. The follicular fluid used to load the oocytes was previously aspirated form follicles (diameter of 3–8 mm) from slaughterhouse ovaries. Subsequently, the system with the needle and the COCs was mounted into the guide, which was positioned in the vaginal fornix toward the same side of the ovary containing the dominant follicle. The needle was then pushed through the vaginal fornix and the follicle wall. When the needle became visible in the follicular antrum, the 60 μL of follicular fluid containing the COCs was injected into the follicle. To induce ovulation, just after the follicular injection, 1 mL of a GnRH analogue (Gestran, Tecnopec, Sao Paulo, Brazil) was administered.

Artificial insemination was performed immediately after IFIOT, using a single dose of frozen-thawed semen from a proven Gir bull in the preexperiment and from the same Nellore bull as was used in the IVF treatments in the main experiment. Between 10 and 25 oocytes were injected into the dominant follicle ($\geq$10 mm) of each recipient for both the experiment and preexperiment.

2.8. Evaluation of follicular diameter and blood vascularization (D0)

On the day of injection (D0), the follicle diameter and vascularization of the dominant follicle were evaluated using a Color Doppler ultrasound (MyLab 30 Vet Gold,

Esaote, Italy) equipped with a 7.5-MHz linear probe. Vascularization was observed and classified according to Matsui et al. [33] as follows: (1) vascularization was absent, and no colored images surrounding the follicle were observed; (2) initial vascularization, between 20% and 40% of the structure was colored; (3) intermediate vascularization, between 41% and 60% of the structure was colored; (4) intense vascularization, between 61% and 80% of the structure was colored; and (5) complete vascularization, between 81% and 100% of the structure was colored.

2.9. Uterine flush and embryo recovery

Eight days after IFIOT of fresh or vitrified and warmed oocytes, the uteri of the recipient heifers were flushed using a Foley catheter (N°. 18) that after positioned the cuff was inflated and pulled firmly against the internal cervical orifice. Subsequently, embryos at D7 of development were flushed out by infusing 1000 mL of PBS, in aliquots, into the uterus and draining the fluid out via a catheter attached to an 80-μm embryo filter. Embryos were evaluated under a stereomicroscope and classified according the developmental stage. The number of total structures recovered was recorded excluding one structure or blastocyst from each female, and the embryos were classified as unfertilized oocytes or cleaved, which included those degenerated with more than 2 cells and those developed to morula (Mo), early blastocyst (Bi), blastocyst (Bl), and also expanded blastocyst (Bx) stage.

2.10. Embryo transfer and pregnancy diagnosis

All 11 embryos, recovered from heifers that had undergone IFIOT were transferred one per synchronized recipient, in 0.25 mL of HM to the uterine horn ipsilateral to the CL. Each week, five Nellore heifers were synchronized as described previously for IFOT recipients. Seven days after heat, the recipients that had a CL with a diameter larger than 16 mm were used to receive the embryos.

2.11. Vitrification and warming

Oocyte vitrification was performed as previously described [1] with slight modifications. The HM, which was used to handle oocytes during vitrification and warming, was composed of HEPES-buffered TCM-199 (Gibco) supplemented with 20% fetal calf serum. For vitrification, the groups were first exposed to an equilibrium solution composed of 7.5% ethylene glycol and 7.5% DMSO for 9 to 15 minutes. Oocytes were transferred to a vitrification solution consisting of 15% ethylene glycol, 15% DMSO, and 0.5 M sucrose in HM. Next, the oocytes were placed into a cryotop device in sets of 3 to 5 under a stereomicroscope (Nikon- SMZ 650), and the device was immediately submerged into liquid nitrogen; the time between the second solution exposition and N_2 was fixed between 45 and 90 seconds. Warming was performed immediately after vitrification by immersing the cryotop end for 1 minute into a drop of HM that was prewarmed to 37 °C and supplemented with 1 M sucrose. The oocytes were transferred to HM supplemented with 0.5 M sucrose for 3 minutes and finally to the original HM. Afterward, the oocytes were placed into culture for maturation and subsequent IVF and embryo development.

2.12. Total cell number (Hoechst 33342) and apoptotic cell ratio (terminal deoxynucleotidyl transferase dUTP nick end labeling)

To determine the total cell number and apoptotic cell ratio, expanded embryos were stained with terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and Hoechst 33342. Blastocysts were washed in warm PBS (Life Technologies) supplemented with polyvinylpyrrolidone (PVP; 1 mg/mL) before fixation in 4% paraformaldehyde for 1 hour. All incubation steps occurred at room temperature in the dark unless otherwise noted, and embryos were washed in 1 mg/mL of PVP between each incubation step. After washing in 1 mg/mL of PVP, the blastocysts were incubated in 0.5% Triton-X for 60 minutes. Subsequently, positive (artificial DNA denaturation, TUNEL, and Hoechst 33342 staining) and negative (artificial DNA denaturation, Hoechst staining) controls were incubated with 50 U/mL DNase (Roche, Vilvoorde, Belgium) for 1 hour. Blastocysts (except negative controls) were then stained with a TUNEL enzyme–labeling mix (Roche) for 60 minutes at 37 °C and Hoechst 33342 staining for 10 minutes. Finally, the blastocysts were washed in PVP, mounted on glass slides, and observed under a fluorescent microscope. For each blastocyst, the individual total cell number (blue nuclei, Hoechst 33342) and the total number of apoptotic cells (green nuclei, TUNEL) were determined. The apoptotic cell ratio was defined as the percentage of apoptotic cells of the total cell number.

2.13. Statistical analyses

All data were analyzed using Statistical Analysis System software (SAS, 1999). Cleavage, fertilization, and blastocyst development were compared according by the Kruskal-Wallis test ($P < 0.05$). For follicular and luteal parameters, total cell and apoptotic cell number ANOVA were used, and the means were compared by the Tukey test ($P < 0.05$).

3. Results

3.1. Efficiency of IFIOT

Eight days after IFIOT (D7), the number of structures recovered after uterine flush was compared to the number of oocytes transferred. For each recipient, one developed embryo was assumed to be derived from the recipient's own follicle. Therefore, we analyzed the data excluding one structure or embryo. When only the extra embryos were considered, the overall recovery rate of transferred oocytes was 21% and of those, 26% (5.5% of the transferred oocytes) were morula or blastocyst (Table 1).

All 11 embryos recovered from heifers that had undergone IFIOT were transferred, and four pregnancies (36%) were achieved (Table 2). From one recipient, five embryos were recovered (Table 1); after embryo transfer

Table 1
Number (N) of injected oocytes at Day 0, number and percentage of each recovered structure, stage of development of the recovered embryos, total recovery rate, and recovered rate after exclusion of the recipient possible embryo (extra) in each follicular transfer.

Injection reference	Injected oocytes (n)	Recovery structures (D7)						Total recovery rate (D7)		Extra recovery rates (D7)		
		UFO	Deg	Mo	Bi	Bl	Bx	Structures	Embryos[a]	Structures	Embryos/recovered structures[a]	Embryos/injected oocytes[b]
								n (%)	N (%)	n (%)	n (%)	n (%)
1	22	2	—	—	—	—	1	3 (13.6)	1 (33.3)	2 (9.1)	0 (0.0)	0 (0.0)
2	22	1	5	—	1	—		7 (31.8)	1 (14.3)	6 (27.3)	0 (0.0)	0 (0.0)
3	10	—	—	1	—	—	2	3 (30.0)	3 (100.0)	2 (20.0)	2 (66.7)	2 (20.0)
4	10	—	—	—	—	—		0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
5	22	—	6	—	1	—		7 (31.8)	1 (14.3)	6 (27.3)	0 (0.0)	0 (0.0)
6	22	—	3	—	—	—	5	8 (36.4)	5 (62.5)	7 (31.8)	4 (50.0)	4 (18.2)
Total	108	3	14	1	2	0	8	28 (25.9)	11 (39.3)	23 (21.3)	6 (26.1)	6 (5.6)

Abbreviations: Bi, early blastocyst; Bl, blastocyst; Bx, expanded blastocyst; Deg, degenerated; Mo, morula; UFO, unfertilized oocytes.
[a] The embryos rate was calculated as the ratio of embryos to recovered structures, both in the total and extra embryo columns.
[b] The embryos rate was calculated as the ratio of the total extra embryos to the total injected oocytes.

of these embryos, three pregnancies were obtained (Table 2). Considering that one embryo was originated from the recipient's own oocyte and that three pregnancies were obtained from the same IFIOT, we can assume that at least two embryos were originated from IFIOT oocytes. This recipient had ovulated only one follicle, based on the criteria we used for recipient selection (only one dominant follicle), and the CL examination by ultrasonography.

3.1.1. *Recovery rates of fresh and vitrified immature oocytes after IFIOT*

Table 3 describes the individual recovery of all the recipients from Fresh-IFIOT or Vit-IFIOT. It can be noted that for fresh oocytes, at least two structures were recovered from all the recipients, and in 7 of the 10 replicates (recipient), it was possible to recover at least one extra embryo. In contrast, no extra embryos were recovered from the vitrified oocytes. As presented in the Table 4, the sizes and vascularization of the follicles that received the oocytes were similar ($P > 0.05$) for both groups. Consequently, the CL formed after ovulation was also similar between them ($P > 0.05$). However, the recovery rate of the Vit-IFIOT group (12.0%) was lower than that observed for the Fresh-IFIOT group (47.6%). After uterine flush, 31 extra blastocyst were recovered, corresponding to 12.9% of total injected oocytes (Table 4).

Table 2
Stage of development, grade, and pregnancy diagnosis of each Day-7 embryo produced by follicular transfer of immature oocytes.

Embryo transfer	Injection reference	Embryo (quality)	Pregnancy at 60 days	Pregnancy at 90 days
1	1	Bi (2)	—	—
2	2	Bx (1)	—	—
3	3	Bx (1)	—	—
4	3	Bx (1)	—	—
5	3	Mo (1)	+	+
6	5	Bi (2)	—	—
7	6	Bx (1)	—	—
8	6	Bx (1)	+	+
9	6	Bx (1)	+	+
10	6	Bx (1)	+	+
11	6	Bx (1)	—	—

Abbreviations: Bi, early blastocyst; Bx, expanded blastocyst; Mo, morula.

3.1.2. *Embryo production from fresh or vitrified warmed oocytes produced by the IVF or IFIOT technique*

Embryo production for the IFIOT group was calculated considering the total recovered structures after uterine flushing of recipient's embryo. Cleavage rate was higher ($P < 0.05$) in fresh (81.0%) than in vitrified oocytes (17.3%) for *in vitro* system. Blastocyst development at D7 were higher ($P < 0.05$) for both fresh groups, Fresh-Vitro (42.1%) and Fresh-IFIOT (12.9%), compared with the vitrified groups, Vit-Vitro (6.3%) and Vit-IFIOT (0%). The proportion of recovered blastocysts that were categorized as expanded did not differ ($P > 0.05$) between Fresh-IFIOT and Fresh-Vitro (71%–74%). Both were significantly higher than Vit-Vitro ($P < 0.05$; Table 5).

3.1.3. *Assessment of blastocyst quality by evaluation of total and apoptotic cell number and ratio*

On D7 after IVF or IFIOT, the expanded blastocysts were analyzed for cell number and apoptotic cells. Although lower rates of development were observed for vitrified and warmed oocytes (Table 5), no differences ($P > 0.05$) with respect to total cell number or apoptotic cell ratio were detected among the three treatments (Table 6).

4. Discussion

Cryopreservation of female germplasm is as essential as male germplasm cryopreservation in breeding programs and genetic conservation. Despite its importance, oocyte cryopreservation remains inefficient in most farm animals, such as swine [34,35], ovine [36,37], and cattle [38–40] species. Several critical points of oocyte cryopreservation have been identified, and researchers have attempted to create alternatives to overcome those problems. One factor that may affect oocytes and subsequent embryo development is the *in vitro* system. Therefore, we have attempted to use an entire *in vivo* system by injecting immature oocytes into a preovulatory follicle and allowing all the steps of embryo production to occur *in vivo*. Subsequently, we used that system in an attempt to improve the embryo production from vitrified oocytes. To the best of our knowledge, this is the first time that IFIOT has been used to investigate the effect of embryo production system (*in vitro* vs. *in vivo*)

Table 3
Number (N) of injected oocytes from Fresh-IFIOT and Vit-IFIOT groups.

Group	Recipient	Injected oocytes	Recovery (D7)					Total recovery (D7)		Extra blastocyst[a]
			UFO	Deg	Bi	Bl	Bx	Structures	Blastocyst	
Fresh-IFIOT	1	25	7	8	1	—	6	22	7	6
Fresh-IFIOT	2	25	3	4	1	—	5	13	6	5
Fresh-IFIOT	3	25	1	5	—	—	4	10	4	3
Fresh-IFIOT	4	25	4	8	—	—	—	12	0	0
Fresh-IFIOT	5	25	—	4	—	—	1	5	1	0
Fresh-IFIOT	6	25	6	8	1	3	1	19	5	4
Fresh-IFIOT	7	25	3	6	—	—	—	9	0	0
Fresh-IFIOT	8	25	3	7	—	1	7	18	8	7
Fresh-IFIOT	9	25	1	3	2	—	4	10	6	5
Fresh-IFIOT	10	15	2	3	—	—	2	7	2	1
Vit-IFIOT	11	25	—	1	—	—	1	2	1	0
Vit-IFIOT	12	25	3	4	—	—	—	7	0	0
Vit-IFIOT	13	25	1	—	—	—	—	1	0	0
Vit-IFIOT	14	25	14	—	—	—	—	14	0	0
Vit-IFIOT	15	25	—	—	1	—	—	1	1	0
Vit-IFIOT	16	25	—	—	1	—	—	1	1	0
Vit-IFIOT	17	25	3	4	—	—	—	7	0	0
Vit-IFIOT	18	25	—	1	—	—	1	2	1	0
Vit-IFIOT	19	25	—	—	—	—	1	1	1	0
Total										
Fresh-IFIOT	10	240	30	0	5	4	30	125	39	31
Vit-IFIOT	9	225	21	10	2	0	3	36	5	0

Number recovered structures and blastocysts at different developmental stage. Total recovery number of structures, blastocyst and extra blastocyst at D7. Abbreviations: Bi, early blastocyst; Bl, blastocyst; Bx, expanded blastocyst; Deg, degenerated; IFIOT, intrafollicular immature oocyte transfer; Mo, morula; UFO, unfertilized oocytes.

[a] The extra blastocyst is presented excluding one, considered to be from the recipient oocyte.

on blastocyst development of vitrified-warmed oocytes. Before using vitrified and warmed COCs for intrafollicular transfer, we performed a preexperiment using COC collected by OPU. Overall, only 21% of transferred oocytes were recovered from the uterus, of which 26% were embryos meaning that the overall embryo (Mo, Bi, Bl, or Bx) rate per transferred oocytes was 5.6%. Those results are close to that reported by Kassens et al. [29] using *in vitro*–matured oocytes, in which overall embryo (Mo, Bi, Bl, or Bx) production per transferred oocyte was 8.4%.

The results showed that even though the total flushing outcome was low, five of six injected heifers yielded more than one recovered structure. If we considered that one of those structures was from the recipient itself, at least two extra structures were recovered from each of the five recipients suggesting that those extra structures were originated from IFIOT COCs. After all the six IFIOTs performed, 11 grade I and II embryos were transferred to recipients, and 60 and 90 days later, four pregnancies were detected by ultrasonography, of which two may be safely assumed to be from IFIOT. Genotype analyses will be performed to confirm which of the pregnancies resulted from IFIOT-derived embryos. Thus, we confirmed that the system worked for immature oocytes and therefore could be tested for vitrified oocytes. There are several factors that can be responsible for the low recovery rate observed in our study in which 21% of transferred oocyte recovered from the uterus. Among the possible factors, we can point out that some oocytes could have been lost during the injection and that some transferred oocytes could have lost cumulus cell during injection, decreasing the percentage of COCs captured by the oviduct. It is important to highlight that the low recovery rate is a common finding in all few studies published to date using this technique.

One of the observations made by Kassens et al. [29] was that the follicle diameter at the moment of the injection was very important for the amount of extra embryos recovered. In our case, to achieve synchrony between the

Table 4
Measurement of the diameter and vascular irrigation of follicles at Day 0 and corpus luteum at Day 7.

Treatment	Replicates	Injected oocytes	Follicle at D0		Corpus luteum at D7		Recovery at D7		
	n	n	Diameter (mm), mean ± SD	Irrigation, mean ± SD	Diameter (mm), mean ± SD	Irrigation, mean ± SD	Extra structures, n (% ± SD)	Extra blastocyst/recovered structures, n (% ± SD)	Extra blastocyst/injected oocytes, % ± SD
Fresh	10	240	12.6 ± 0.9	1.6 ± 0.6	19.0 ± 1.9	4.8 ± 0.4	115 (47.6 ± 19.7)[a]	31 (24.8 ± 16.8)[a]	12.9 ± 9.8[a]
Vit	9	225	13.1 ± 0.6	1.6 ± 0.5	19.5 ± 2.2	4.8 ± 0.3	27 (12.0 ± 16.9)[b]	0 (0.0)[b]	0.0[b]

Recovery rates of total structures and extra embryos (after exclusion of recipient embryo) at Day 7 of development, from fresh or vitrified and warmed oocytes, after intrafollicular immature oocyte transfer.

[a,b] Values with different superscripts differ significantly according to ANOVA and the Tukey test ($P < 0.05$).

Table 5
Developmental rates at Day 7 of fresh or vitrified and warmed (Vit) oocytes after *in vitro* production (Vitro) or intrafollicular immature oocyte transfer (IFIOT).

Treatment	n	Blastocyst at D7	Expanded blastocyst at D7[f]
		n (%)	n (%)
Fresh-Vitro	190	80 (42.1)[a]	57 (71.2)[a]
Vit-Vitro	173	11 (6.3)[c]	4 (36.3)[c]
Fresh-IFIOT[e]	240	31 (12.9)[b]	23 (74.2)[a]
Vit-IFIOT[e]	225	0 (0.0)[d]	0 (0.0)[d]

[a,b,c,d]Values with different superscripts in the same column are significantly different by Kruskal-Wallis ($P < 0.05$).
[e] Blastocyst rates at D7 was calculated after exclusion of the most developed blastocyst (considered the recipient blastocyst).
[f] The blastocyst expansion rate was calculated as the ratio of the expanded to the total of blastocyst at D7.

recipient follicle and transferred oocytes, the follicle needed to be at least 18 hours ahead of the expected moment of ovulation to provide sufficient time for IVM to occur. Then, in the experiment 2 to control the effect of the follicle stage on embryo recovery, we evaluated both the follicle diameter and the follicular blood vascularization based on the fact that the proximity to ovulation is correlated with increasing blood vascularity [20,33]. Therefore, to ensure that the follicles were not too close to the ovulation time, we only used those that exhibited initial vascularization (20%–40% of vascularization). As we fixed the size and vascularization of the follicles, no differences in those characteristics were observed between the two types of oocytes injected, fresh or vitrified, and we could eliminate the effect of the follicle on the number of embryos recovered.

In experiment 2, the total recovery rate after IFOT, excluding the recipient oocyte obtained after fresh oocytes injection, was 47.6%. The quantity of structures recovered was higher than the 35% reported by Kassens et al. [29] or 32% obtained by Bergfelt et al. [27]. In addition, the recovery rate of embryos at the Bi, Bl, or Bx stage was even higher in our study (12.9%) compared to that 3.2% reported by Kassens et al. [29]. Other possible explanation could be the number of oocytes because instead of injecting 60 oocytes [29], we have injected a maximum of 25 oocytes in each follicle, facilitating the capture of oocytes by the oviduct fimbriae during ovulation. In addition, follicle selection could also be responsible for the good results, once we have fixed the diameter and status of vascularization before using the follicle for oocyte injection. However, when we compared the results from our first experiment in which we used the same number of oocytes and same follicle selection, it could be suggested that the main factor influencing recovery rate is oocyte quality.

When fresh and vitrified/warmed oocytes were compared, the total flushing outcome was lower for vitrified (12.0%) than for fresh oocytes (47%). It is very likely that vitrification at the germinal vesicle stage may affect not only the oocyte viability but also the cumulus cells in some way such that cumulus expansion became compromised. Expansion is essential to facilitate oocyte capture by the ciliated epithelial cells of the infundibulum [41] and its transport to the fertilization site; therefore, if this process was altered, it could affect the recovery rates. The quantity of structure retrieved after IFIOT was higher for the fresh than for vitrified oocytes as was the percentage of those that had developed to the blastocyst stage. These results could be easily explained by the damages caused by vitrification that cannot be overcome, even with the maturation occurring inside the follicle. The differences on recovery and extra blastocyst observed in the experiments 1 (5.6%) and 2 (12.9%) were, probably, related to type of COCs injected. In the preexperiment, COCs were obtained by OPU. Whereas for Fresh-IFIOT, COCs were obtained from slaughterhouse ovaries that provide large number of COCs allowing us to do a more strict selection and to end with higher quality oocytes.

Both the blastocyst development and the blastocyst quality were compared between groups. *In vivo* embryo quality is affected by uterus status [42]. At D7, the uterus must be under the influence of progesterone, produced by a healthy CL. Therefore, we evaluated the CL immediately before uterine flushing, and no difference was observed between the groups. The size and vascularity of the CL were similar to those previously described for a healthy CL at D7. This observation suggests that the injection did not alter the CL formation and development and that only one CL was present.

In addition to the possibility of producing embryos from fresh and vitrified immature oocytes by transfers to follicles, we also want to compare the quality characteristics of those embryos. The most effective tool to accesses embryo quality would be to transfer them to a recipient and to observe pregnancy and birth [29]. Because of the difficulties and cost to maintain the recipients, we compared the total cell number (Hoechst) and the total number of apoptotic cells (TUNEL) in the expanded blastocyst at D7 obtained from all the treatments. No differences were observed among the treatments with respect to the total number or number of apoptotic cells, suggesting that IFOT-derived Bx had the same morphological characteristics of those produced *in vitro*. We did not recover any extra embryo from Vit-IFIOT, and thus, we cannot draw any conclusion regarding those characteristics. Because Zhao et al. [43], as well as a number of others, have demonstrated that the *in vitro* system elevates reactive oxygen species status, which can induce apoptotic pathways [44], we had

Table 6
Assessment of the number and standard deviation (n ± SD) of total and apoptotic cell numbers of expanded blastocyst at D7, after *in vitro* production (vitro) or intrafollicular immature oocyte transfer (IFIOT), with fresh or in vitrified and warmed oocytes.

Treatment	Expanded blastocyst D7 (Bx)	Cell number		Apoptotic/total cell
	n	Total	Apoptotic	Ratio %
		n ± SD	n ± SD	
Fresh-Vitro	47	135.0 ± 17.2	4.9 ± 2.9	3.6%
Vit-Vitro	4	135.3 ± 6.8	3.8 ± 2.6	2.8%
Fresh-IFIOT[a]	17	132.1 ± 9.3	4.7 ± 2.1	3.5%
Vit-IFIOT[a]	0	—	—	—

Values compared by ANOVA and Tukey test ($P < 0.05$).
[a] Expanded blastocyst at Day 7 after exclusion of the most developed blastocyst (considered the recipient blastocyst).

expected that the embryo quality would be better in the *in vivo* cultured after IFIOT. However, it is important to highlight that we only use cell number and number of apoptotic cells to evaluate the quality; it is possible that the results may have differed if we had used other variables.

In summary, this technique of immature bovine oocytes injection resulted in viable blastocysts at D7 and pregnancies at 60 and 90 days. However, no improvement on efficiency of embryo production from vitrified oocytes in the germinal vesicle stage was observed.

The IFOT procedure remains less effective than *in vitro* embryo production, both because of the poor rate recovery of ova/embryos from the uterus (21%–48%) and the low rate of blastocyst development per recovered structure (25%–26% vs. 42%) for *in vitro* embryo production. Summarizing, the technique produces 12.9% of embryo with respect to the total injected oocytes, equivalent to one-third of the embryos produced by IVP. Further investigation into factors related to the success of IFOT is needed for it to become an effective, repeatable procedure. If effective, the IFIOT technique would open a wide range of possibilities for breeding programs and system for research because it allows the production of a high number of embryos in a totally *in vivo* system without any hormonal super stimulation or extensive laboratory facilities.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2016.07.003.

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